Exhibit 9

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CD91 Is a Common Receptor for Heat Shock Proteins gp96, hsp90, hsp70, and Calreticulin

Sreyashi Basu, Robert J. Binder,
Thirumalai Ramalingam,
and Pramod K. Srivastava*
Center for Immunotherapy of Cancer
and Infectious Diseases
University of Connecticut School of Medicine
MC1601
Farmington, Connecticut 06030

Summary

Complexes of the heat shock protein gp96 and antigenic peptides are taken up by antigen-presenting cells and presented by MHC class I molecules. In order to explain the unusual efficiency of this process, the uptake of gp96 had been postulated to occur through a receptor, identified recently as CD91. We show here that complexes of peptides with heat shock proteins hsp90, calreticulin, and hsp70 are also taken up by macrophages and dendritic cells and re-presented by MHC class I molecules. All heat shock proteins utilize the CD91 receptor, even though some of the proteins have no homology with each other. Postuptake processing of gp96-chaperoned peptides requires proteasomes and the transporters associated with antigen processing, utilizing the classical endogenous antigen presentation pathway.

Introduction

Purified preparations of heat shock proteins (HSPs) hsp70 and hsp90 of the cytosol and gp96 and calreticulin (CRT) of the endoplasmic reticulum have been shown to elicit antigen-specific cellular immunity upon immunization (reviewed in Srivastava et al., 1998; Basu and Srivastava, 1999). The observed immunogenicity of HSP preparations has been shown to derive from the antigenic peptides chaperoned by the HSPs. HSP-peptide complexes can also be reconstituted in vitro by complexing a given peptide with hsp70, gp96, or CRT (Blachere et al., 1997; Basu and Srivastava, 1999), and such complexes are immunogenic in the same manner as the HSP-peptide complexes generated in vivo. As little as a few picograms of antigenic peptides are immunogenic if complexed to the HSPs but not to non-HSP peptide binding proteins such as albumin (Blachere et al., 1997). Further, the immunogenicity of gp96-peptide complexes has been shown to be dependent on the presence of functional antigen-presenting cells (APCs), and abrogation of their function renders mice incapable of being immunized with gp96-peptide complexes (Udono et al., 1994). The above two observations led to the suggestion that APCs express receptors for HSPs and that the receptors are responsible for the extremely high adjuvanticity of the HSPs (Srivastava et al., 1994). We have recently identified the α_2 macroglobulin receptor CD91 as the receptor for gp96 (Binder et al., 2000a); gp96 molecules bind directly to CD91, and antibodies to CD91 inhibit re-presentation of gp96-chaperoned peptides by APCs. Further, α_2 macroglobulin, a previously known ligand for CD91, inhibits re-presentation of gp96-chaperoned peptides by APCs. The mechanisms through which HSPs other than gp96 elicit immune responses have not been defined explicitly, although it was originally expected that broadly similar mechanisms must be involved. Indeed, Castellino et al. have shown recently that hsp70-peptide complexes are also taken up through a receptor-like mechanism (Castellino et al., 2000).

The variety of HSPs that can chaperone peptides and immunize, as well as the broad array of peptides that they can chaperone, has been a continuing strength, as well as a riddle, in the understanding of this pathway. We report here certain observations that provide a common mechanism through which various HSPs chaperoning an extremely small quantity of peptides elicit antigen-specific immunity. We show that re-presentation of HSP-chaperoned peptides by APCs functions in a broadly similar manner for all HSPs tested, that all HSPs tested appear to utilize a common CD91 receptor, and that all HSPs tested can utilize a variety of APCs at various stages of differentiation (including an immortalized cultured macrophage line), each of which can re-present HSP-chaperoned peptides. These studies also show that re-presentation of gp96-chaperoned peptides, like that of hsp70-chaperoned peptides, follows the classical proteasome-dependent, transporter associated with antigen processing (TAP)-dependent pathway of presentation of endogenous antigens by MHC class I molecules.

As HSPs are released from cells as a result of necrotic but not apoptotic death (Basu et al., 2000), their ability to mediate re-presentation of antigens by APCs (Suto and Srivastava, 1995) potentially provides a general pathway through which antigens of cancers or infectious agents from parenchymatous cells are cross-presented by the APCs to naive T cells in the lymph nodes (Bevan, 1976). An increased understanding of this process may therefore open the way to a deeper revelation of fundamental immunological processes.

Results

Binding of Fluorescence-Labeled HSPs and α_2 Macroglobulin to a Panel of Primary and Cultured Cells

FITC-labeled HSPs, gp96, hsp90, or hsp70, or control non-HSP serum albumin (SA) were incubated with primary cells (peritoneal macrophage, bone marrow–derived dendritic cells) or immortalized cell lines (RAW264.7, RAW 309Cr.1 macrophage, P815 mastocytoma, YAC-1 lymphoma, EL4 thymoma, Meth A and PS-C3H fibrosarcomas, B16 melanoma, CT26 colon carcinoma, and UV6139 squamous cell carcinoma), as described in Experimental

*To whom correspondence should be addressed (e-mail: srivastava@nso2.uchc.edu).

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Table 1. Specific Binding of HSPs and α_2 Macroglobulin to Primary Cultures and Cell Lines of Several Histological Origins[a]

			[b]Percentage of Cells Binding with FITC-Labeled:				
Cells	Cell Type	Haplotype	α_2M	gp96	hsp70	hsp90	SA
B16	melanoma	b	0.1	3.5	6.4	8.0	0.3
CT26	carcinoma	d	ND	0.3	3.1	5.5	0.4
YAC-1	lymphoma	b	0.1	3.1	23.0	5.0	0.2
EL4	T cell thymoma	b	0.1	2.9	3.0	6.6	1.0
Meth A	sarcoma	d	0.1	0.1	1.5	0.9	0.5
PS-C3H	fibrosarcoma	k	0.1	0.1	2.0	0.3	0.3
UV6139	sarcoma	k	11	0.0	0.7	0.2	1.5
P815	mastocytoma	d	0.1	1.1	1.7	0.7	0.2
Peritoneal cells	macrophage	d	90	97	82	82	11
BM-DCs	dendritic cells	b and d	+[d]	+[d]	+[d]	+[d]	–
RAW264.7[c]	macrophage	d	76	82	85	90	8.0
RAW309Cr.1[c]	macrophage	b × d	0.1	0.1	0.1	0.1	0.1

[a] Cells were incubated with FITC-labeled α_2M, gp96, hsp70, hsp90, or SA. After removal of excess protein by extensive washing, cells were analyzed for FITC staining by flow cytometry.
[b] Indicates percentage of cells staining with FITC over background staining with FITC alone.
[c] See Figure 1.
[d] Cells were examined by confocal microscopy. All CD11c$^+$ cells were intensely positive for binding to the three HSPs and α_2M.

Procedures. After removal of unbound protein by extensive washing, cells were analyzed by flow cytometry. As shown in Table 1, the peritoneal macrophages and the bone marrow–derived dendritic cells showed robust binding of each of the three HSPs but not albumin. However, of the two macrophage cell lines, only one of them, RAW264.7, bound the three HSPs. RAW309Cr.1 did not bind any of the HSPs (Figures 1A and 1B). Out of eight other cell lines tested with the FITC-labeled gp96, hsp90, and hsp70, none was observed to bind to HSP in a manner comparable to the binding observed with RAW264.7. YAC-1 was observed to bind hsp70 but only to a significantly smaller degree. The binding was only a fraction of that observed with APCs.

CD91 or the α2 macroglobulin receptor has been identified recently as the receptor for gp96. All of the cell types in Table 1 were also tested for the presence of CD91 by staining with FITC-α2 macroglobulin. CD91 showed precisely the same pattern of distribution as did each of the three HSPs (Table 1).

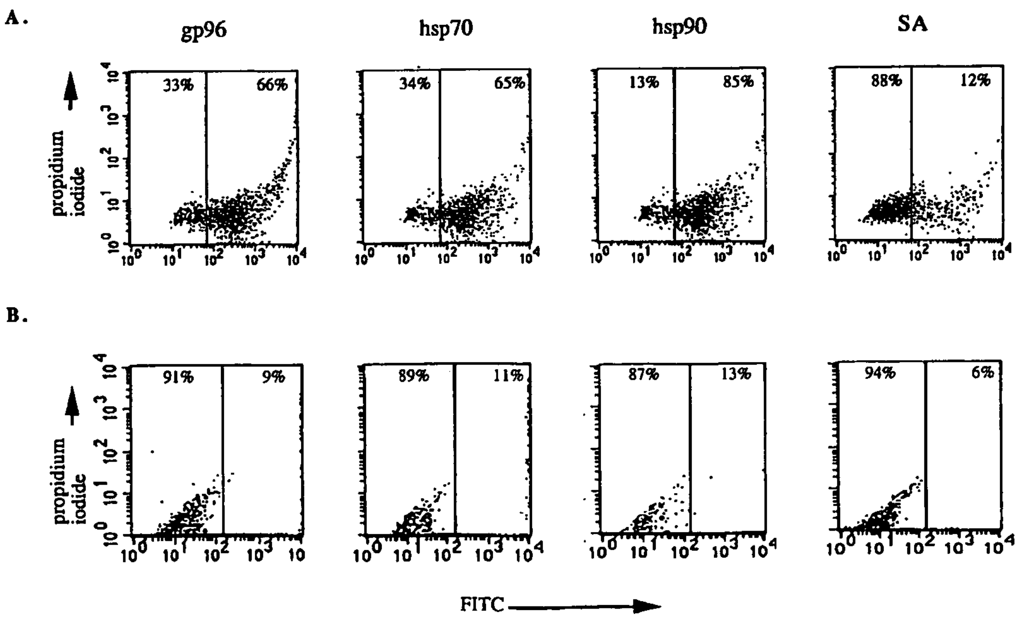

Figure 1. RAW264.7 but Not RAW309Cr.1 Binds Specifically to HSPs
The macrophage cell lines RAW264.7 (A) or RAW309Cr.1 (B) were incubated with 100 μg/ml of FITC-labeled gp96, hsp90, hsp70, or SA. Excess protein was removed by extensive washing. Cells were analyzed by flow cytometry for the presence of FITC-labeled cells. Live cells only were gated based on FSC.

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The Ability of Cells to Bind HSPs and α_2M Correlates with the Ability to Re-Present gp96-Chaperoned Peptides

We tested if the ability of a particular cell type to bind HSPs or α_2 macroglobulin, as shown in Table 1, correlates with its ability to re-present gp96-chaperoned peptides. Re-presentation studies are done typically by incubating APCs and an HSP, chaperoning a known peptide, with T cells specific for an epitope present in the chaperoned peptide (Suto and Srivastava, 1995). The experimental system is set up such that the peptide cannot charge directly onto MHC class I but requires intracellular processing followed by presentation to T cells. VSV8 and AH1 antigenic systems were used in these studies. The VSV8 epitope (RGYVYQGL) is presented by the K^b molecule, and VSV19 (SLSDL RGYVYQGLKSGNVS) is its extended variant, which cannot charge K^b directly. AH1 (SPSYVYHQF) is an L^d-restricted epitope of a murine leukemia virus envelope protein gp70 (Huang et al., 1996), and AH1/19 (RVTYHSPSYVYHQFERRAK) is its extended version. Peritoneal macrophage and BM-DCs were tested side by side for re-presentation in the VSV8 system, and both cell types were able to re-present gp96-chaperoned VSV19 to VSV8-specific T cells (Figure 2A). EL4 and B16 cells, both of the b haplotype, were also tested and were found unable to re-present in identical assays (data not shown). The BM-DCs appear to re-present gp96-chaperoned VSV19 better than macrophage did, although the difference may not be significant. Further, it is not possible to determine from the data if this difference derives from the better T cell stimulatory properties of DCs in general or whether the DCs are specifically more efficient than macrophage at re-presenting gp96-chaperoned peptides. The two macrophage cell lines RAW309Cr.1 and RAW264.7 were tested for their re-presentation ability in the AH1 system. In parallel with the HSP and α2M staining data (Table 1), RAW264.7 cells but not RAW309Cr.1 cells were observed to be capable of re-presenting gp96-chaperoned AH1 peptides (Figure 2B).

We compared the gp96 binding and the re-presenting ability of immature and mature DCs (Figure 3). Cultures of freshly isolated immature BM-DC were pulsed with medium alone or LPS as a maturation signal. The cultures were stained for gp96 binding and for expression of CD40 and CD11c. As expected, CD40 expression in the mature DC cultures was significantly higher than in the immature DC cultures, while CD11c expression was unchanged (Figure 3A, top). Comparison of gp96 binding between immature and mature DC cultures, on the other hand, failed to show any differences between the two populations (Figure 3A, bottom). The immature and mature BM-DC cultures were also tested functionally for their ability to re-present gp96-chaperoned AH1/19 and were found to be similar in that respect (Figure 3B). The results from gp96 binding and functional re-presentation studies were thus consistent with each other.

Peptides Chaperoned by hsp90, hsp70, and CRT Are Re-Presented by MHC Class I Molecules of APCs

gp96 was the first HSP for which the re-presentation phenomenon was experimentally shown (Suto and Srivastava, 1995). Hsp70-chaperoned peptides have been shown recently to be re-presented by APCs (Castellino et al., 2000). The ability of other HSPs, hsp90, and CRT to introduce chaperoned peptides into the endogenous presentation pathway was tested in the AH1 system with RAW264.7 cells as the APCs. RAW264.7 cells were pulsed with hsp90, hsp70, CRT, or gp96, as a positive control, by themselves, or chaperoning the AH1/19 peptide. Chaperoning of peptides by the HSPs was accomplished in vitro as previously described (Blachere et al., 1997; Basu and Srivastava, 1999). T cells specific for L^d/AH1 secreted IFN-γ when the RAW264.7 cells were pulsed with complexes of hsp90, hsp70, CRT, or gp96 with AH1/19 but not when the HSPs were not complexed with the peptide (Figure 4). Pulsing of RAW264.7 cells with AH1/19 alone did not lead to surface loading of L^d molecules and consequent stimulation of T cells. Further, RAW264.7 cells pulsed with complexes of serum albumin with AH1/19 also failed to stimulate L^d/AH1-specific T cells, thus indicating the specific requirement of HSPs for introducing the chaperoned peptides into the endogenous presentation pathway (Figure 4).

gp96, hsp90, hsp70, and CRT Engage a Common Receptor

Does each HSP have a unique receptor, or do they share a common receptor? This question was addressed by three independent criteria: by measuring re-presentation of gp96-chaperoned AH1/19 (as in Figures 2–4) in the presence of excess and titrated quantities of free (i.e., not complexed to AH1/19) gp96, hsp90, hsp70, or serum albumin; by testing if α_2 macroglobulin, a known ligand for CD91, a receptor for gp96, can inhibit re-presentation of peptides chaperoned by gp96, hsp90, hsp70, or CRT; and, finally, if anti-CD91 antibody can inhibit re-presentation of peptides chaperoned by some or all of the HSPs.

The gp96-AH1/19 complex was added to RAW264.7 cultures at a fixed final concentration of 40 μg/ml, while the competing HSPs or serum albumin were added at concentrations between 200 and 800 μg/ml. It was observed (Figure 5A) that all three competing HSPs could inhibit re-presentation of gp96-chaperoned AH1/19, albeit with different efficiencies. gp96 was able to compete with itself, while hsp90 was an even better competitor than gp96. hsp70 was a less efficient competitor than gp96 but was a significant competitor. Albumin competed inefficiently. In quantitative terms, ~2-fold molar excess of hsp90, 6-fold molar excess of gp96, and a 13-fold molar excess of hsp70 were required to inhibit by 50% the re-presentation of gp96-chaperoned peptides at a gp96 concentration of 40 μg/ml. All three HSPs were able to inhibit the re-presentation of gp96-chaperoned peptides completely at the highest concentration tested. This observation suggests that gp96, hsp90, and hsp70 utilize a single receptor, albeit with differing specificities.

In additional experiments, increasing quantities of α_2 macroglobulin were added to re-presentation assays where AH1/19 chaperoned by gp96, hsp90, hsp70, or CRT was re-presented by RAW264.7 cells to L^d/AH1-specific T cells. α_2 macroglobulin was observed to inhibit, in a titratable manner, re-presentation of peptides chaperoned by each of the four HSPs (Figure 5B). Re-presentation of peptides chaperoned by gp96, hsp70,

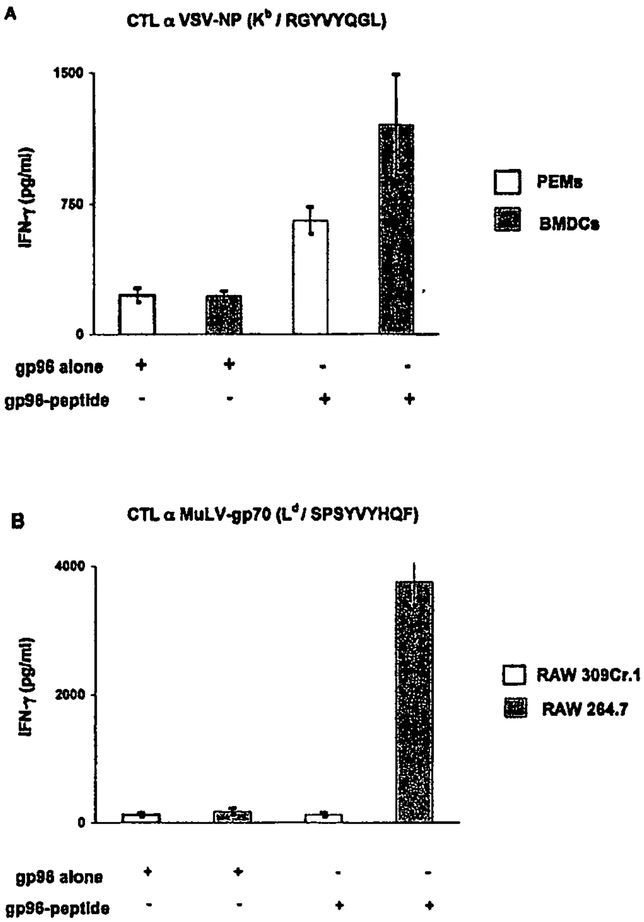

Figure 2. Re-Presentation of gp96-Chaperoned Peptides by APCs that Bind HSPs and α_2 Macroglobulin

(A) Peritoneal macrophage or BM-DCs from C57Bl/6 mice (1 × 10⁴) were pulsed with gp96 (40 μg/ml) by itself or complexed to the VSV19 peptide and used to stimulate the VSV8-specific CTL line (1 × 10⁴) for 20 hr at 37°C. Culture supernatants were tested for the presence of IFN-γ (pg/ml) as a marker for CTL stimulation.

(B) RAW264.7 or RAW309Cr.1 macrophage lines were cultured with gp96 (40 μg/ml) by itself or complexed to the AH1/19 peptide and used to stimulate AH1-specific CTLs (1 × 10⁴) for 20 hr at 37°C. Culture supernatants were tested as in (A).

and CRT was inhibited equally, while re-presentation of hsp90-chaperoned peptide was inhibited the most effectively and almost completely at the highest concentration of α_2 macroglobulin tested. Serum albumin, when tested at the highest concentration, inhibited re-presentation only modestly. It may be noted that, while the data in Figure 5A show that the specific peptide-deficient HSPs inhibited re-presentation of gp96-AH1/19 complexes completely at the highest concentrations tested, α_2 macroglobulin appears far less effective, in quantitative terms, at inhibiting the re-presentation of peptides chaperoned by three of the four HSPs (Figure 5B). However, this quantitative disparity disappears if one notes that the α_2 macroglobulin molecule is ~10 times larger in molecular mass than the average HSP molecule.

A mouse monoclonal anti-CD91 IgG_1 antibody and isotype control antibodies were tested for their ability to inhibit re-presentation of peptides chaperoned by gp96, hsp90, hsp70, and CRT. As before, the RAW264.7/AH1 system was utilized, and the antibodies were added to re-presentation cultures at the concentrations indicated (Figure 5C). Anti-CD91 antibody was observed to inhibit, titratably and specifically, the re-presentation of AH1 chaperoned by each of the four HSPs tested. The isotype control antibody did not inhibit re-presentation in any instance. Further, the inhibition mediated by the anti-CD91 antibody was complete and uniform for each of the HSPs, indicating that CD91 is the sole receptor for each of the four HSPs.

Requirement of a Functional Proteasome Complex for the Re-Presentation of gp96-Chaperoned Peptides by APCs

The re-presentation assay was carried out in the presence or absence of the specific proteasome inhibitor lactacystin. The peritoneal macrophages were treated or untreated with lactacystin for 2 hr and then cultured with gp96-VSV19 complex for another 2 hr in the presence or absence of the inhibitor. The cells were chromium labeled at the same time for 1 hr and then washed and used as targets against CD8⁺ T cells specific for VSV8, in a 4 hr chromium release assay. Gp96-VSV19, lactacystin-untreated pulsed APCs were lysed by VSV8-

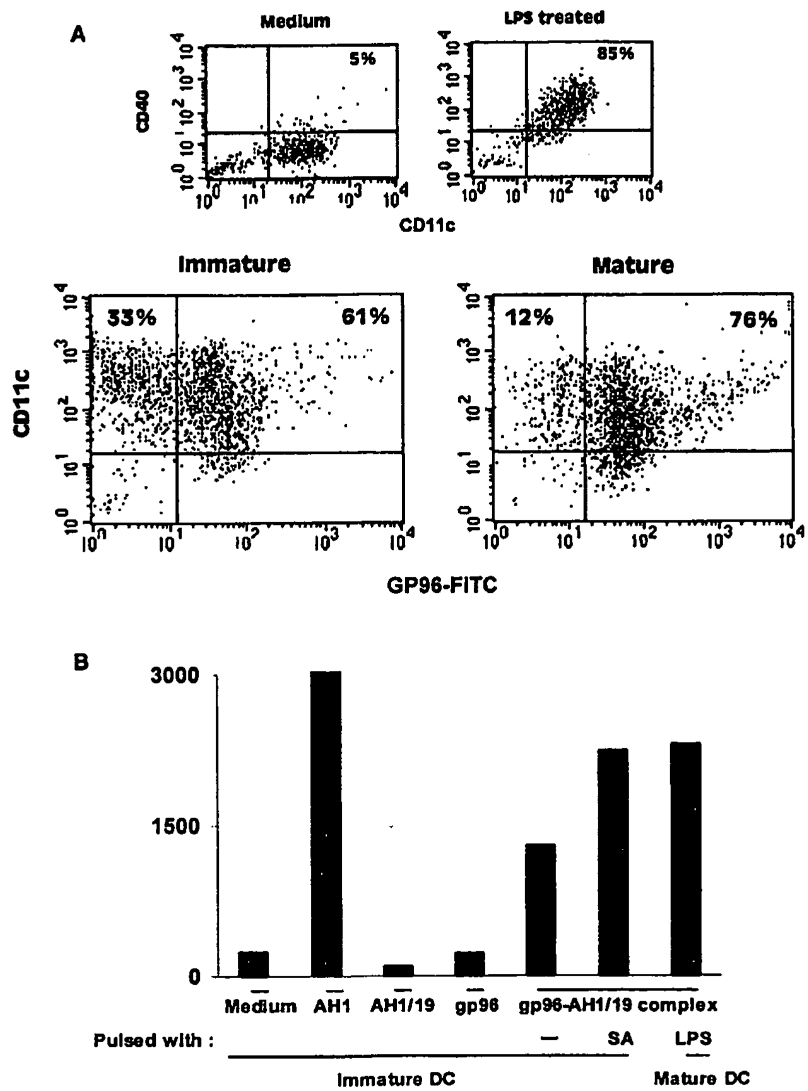

Figure 3. Comparison of Immature and Mature BM-DCs with Respect to Binding of gp96 and Ability to Re-Present gp96-Chaperoned Peptides

(A) (Top) BM-DC cultures in medium without (left) or with (right) LPS were stained for expression of CD11c and CD40. Enhanced expression of CD40 is seen on the mature DC cultures. (Bottom) Both cultures were also stained for gp96 binding and expression of CD11c. No differences were detected between the immature and mature DC cultures.

(B) BM-DC cultures were pulsed with medium alone or LPS as a maturation signal or with serum albumin as a negative control. All cultures were tested for re-presentation of gp96-chaperoned AH1/19 as described in Figure 2.

specific $CD8^+$ T cells (Figure 6A). As observed previously for gp96 (Suto and Srivastava, 1995) and for hsp70 (Castellino et al., 2000), only a small proportion of pulsed APCs were lysed by the APCs, even at the highest E:T ratio tested (Figure 6A). The T cells pulsed with VSV8 (through surface charging) were lysed in a titratable and more significant degree, indicating that the APCs were entirely capable of being lysed. The basis of the selective lysability of APCs re-presenting HSP-chaperoned peptides is still unclear. However, and regardless of this observation, the lactacystin-treated, gp96-VSV19 pulsed APCs were not recognized by the VSV8-specific $CD8^+$ T cells and were not lysed by them (Figure 6A). Inhibition of proteasomal function thus inhibits the processing of peptides chaperoned by gp96. As other HSPs tested also use the same portal of entry (Figure 5), it is assumed that inhibition of proteasome function interferes with processing of peptides chaperoned by them

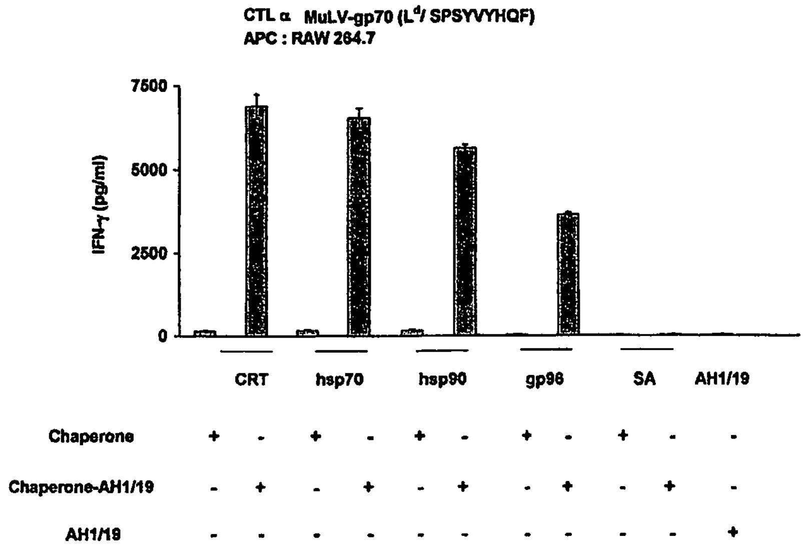

Figure 4. Peptides Chaperoned by hsp90, CRT, hsp70, and gp96 but Not Serum Albumin Are Re-Presented by RAW264.7 Cells
The chaperones, uncomplexed or complexed to the AH1/19 peptide, were used to pulse RAW264.7 cells, which were tested for their ability to stimulate cognate CTLs, as described in the legend to Figure 2B. The unchaperoned AH1/19 did not stimulate the CTLs.

as well. The data recently reported by Castellino et al. (2000) for hsp70 are consistent with this inference.

Re-Presentation of gp96-Chaperoned Peptides by MHC Class I of the APCs Requires a Functional TAP

The requirement of TAP in re-presentation of gp96-chaperoned peptides by APCs was tested. In a re-presentation assay in vitro, gp96 purified from liver or the same gp96 complexed with VSV19 was pulsed onto primary cultures of peritoneal macrophages or BM-DCs derived from $TAP^{+/+}$ or $TAP^{-/-}$ mice. The pulsed macrophage or BM-DCs were used to stimulate CD8$^+$ T cell lines specific for K^b/VSV8. After incubation for 20 hr, the culture supernatants were tested for release of IFN-γ, as a marker for T cell stimulation (Figure 6B). It was observed that APCs from $TAP^{+/+}$ mice stimulated the CD8$^+$ T cells specifically when cultured in the presence of gp96 complexed to VSV19, but APCs from $TAP1^{-/-}$ mice were unable to do so. There were no detectable differences between the data with macrophage and DCs. This result indicates that gp96-chaperoned peptides must enter the endoplasmic reticulum through the TAP molecules for being loaded on the MHC class I molecules. As other HSPs tested also use the same portal of entry (Figure 5), it is assumed that peptides chaperoned by other HSPs also require TAP for re-presentation. Part of the data recently reported by Castellino et al. (2000) for hsp70 are consistent with this inference.

In studies in vivo, $TAP1^{-/-}$ (C57BL/6/SV129J) or wild-type (C57BL/6) mice were immunized with the gp96-VSV19 complexes (50 μg gp96) or VSV19 alone or gp96 alone. Spleen cells of immunized mice were cultured with the VSV8 and tested for cytotoxic activity on [^{51}Cr]EL4 cells or EL4 cells pulsed with the VSV8 peptide as targets. Spleen cells of wild-type (C57BL/6) mice immunized with gp96-peptide complex showed VSV8-specific CTL activity, whereas splenocytes of $TAP1^{-/-}$ mice immunized with gp96-peptide complex showed no cytotoxic activity (Figure 6C). It may be argued that the lack of CTL activity in $TAP^{-/-}$ mice is a result of the poor loading and stability of MHC class I molecules in general, rather than because of a specific block in re-presentation. While this is possible and is difficult to entirely refute, we are easily able to generate VSV8-specific CTLs in $TAP^{-/-}$ mice, as in $TAP^{+/+}$ mice, by immunization with VSV8 peptide in incomplete Freund's adjuvant (Figure 6D). Sandberg et al. (1996) have reported similar data. In any case, the data from re-presentation assays in vitro using APCs from $TAP^{+/+}$ and $TAP^{-/-}$ mice (Figure 6B) demonstrate the TAP requirement for re-presentation without the complexity introduced by the experiment in vivo (Figure 6C).

Discussion

A receptor for HSPs was originally envisaged in order to explain the extraordinary immunogenicity of HSP-peptide complexes; very small quantities of antigenic peptides, if associated with HSPs, could elicit antigen-specific T cell responses in mice, and, in spite of the exogenous administration, the response was CD8$^+$ and MHC class I–restricted (Srivastava et al., 1994). The HSPs that could chaperone peptides and immunize were first restricted to gp96 (Srivastava, 1982; Srivastava and Das, 1984; Srivastava et al., 1986), but soon the list grew longer and came to include hsp90 (Ullrich et al., 1986, Udono and Srivastava, 1994), hsp70 (Udono

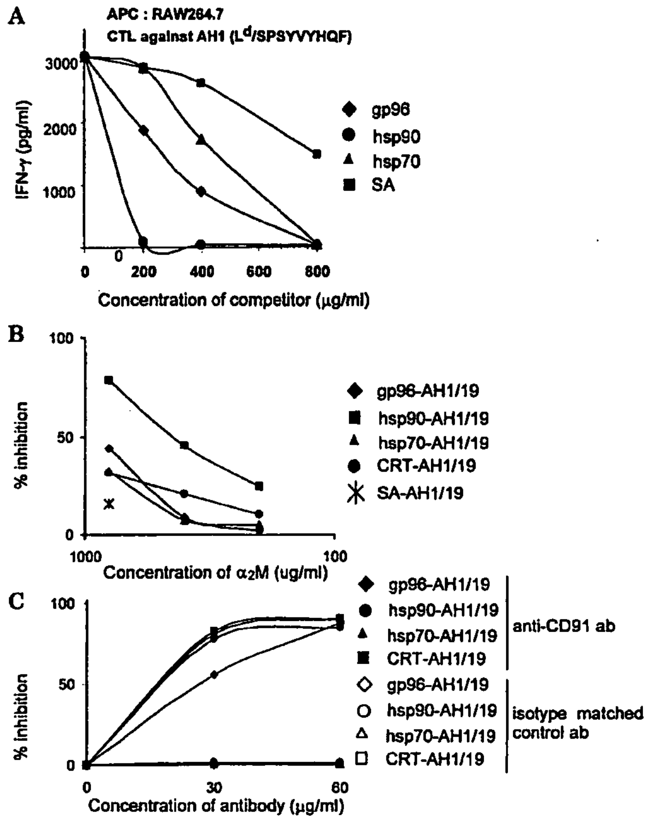

Figure 5. gp96, hsp90, hsp70, and CRT Utilize a Common Receptor for Re-Presentation

(A) RAW264.7 cells were pulsed with gp96-AH1/19 complexes (40 μg/ml gp96) in the presence of increasing concentrations of uncomplexed gp96, hsp90, hsp70, or SA, as indicated. Re-presentation assay was carried out as described in the legend to Figure 2B.

(B) Re-presentation of AH1/19 complexed to gp96, hsp90, hsp70, CRT, or albumin (as described in [A]) was carried out in the presence of increasing concentrations of α2 macroglobulin, a previously known ligand for CD91. As the re-presentation efficiency of each HSP was different (see Figure 4), the data are plotted as percentage inhibition of re-presentation.

(C) Re-presentation of AH1/19 complexed to gp96, hsp90, hsp70, or CRT (as described in [A]) was carried out in the presence of increasing concentrations of anti-CD91 antibody, as indicated. As the re-presentation efficiency of each HSP was different (see Figure 4), the data are plotted as percentage inhibition of re-presentation.

and Srivastava, 1993), CRT (Basu and Srivastava, 1999), and, most recently, hsp110 and grp170 (Wang et al., 2000). The receptor for gp96 was recently identified as CD91, an α_2 macroglobulin receptor. The studies shown here indicate that this receptor is also engaged by hsp90, hsp70, and CRT. This observation is surprising in light of the fact that hsp70, CRT, and hsp90/gp96 have no obvious structural similarities with each other. In another context, HSPs have presented us with this dilemma before: many of the various HSPs have no obvious homologies with each other, and yet they appear to bind many of the same peptides (Breleor et al., 1998; Ishii et al., 1999). It remains to be seen if hsp110, which belongs to the extended hsp70 family, and grpp170, which has no homology to any of the other HSPs, shall share the CD91 receptor. The multiple common properties of the HSPs that share the fourth paradigm (Srivastava, 1994) (i.e., peptide binding, interacting with APCs through a common receptor, ATP binding, and ATPase activity) strongly suggest that these molecules share conformational similarities that are not obvious from their primary sequence. Crystallographic analyses of hsp70 and hsp90 have begun to reveal their structure (Zhu et al., 1996; Prodromou et al., 1997; Stebbins et al., 1997) and shall shed light on this question. An interesting and related question has to do with receptors for HSPs such as hsp60 that do not bind peptides and do not prime T cell responses. The toll-like receptor 4 on APCs has been implicated in such interaction (Ohashi et al., 2000). The role for CD91 in interaction of other HSPs and APCs remains to be examined.

The observations that α_2 macroglobulin and anti-CD91 antibodies inhibit re-presentation by each of the four HSPs completely indicate that CD91 is the only receptor involved in re-presenting peptides chaperoned by any of the four HSPs tested. Considering the increasingly obvious role that the HSPs play in innate (Basu et al., 2000) and adaptive immune response, this observation is somewhat counterintuitive. However, the data on complete inhibition by two independent means (Figure 5) are compelling. We have noticed earlier and we report here significant differences between hsp70 and hsp90/gp96 in their ability to compete for binding to gp96 receptors (Binder et al., 2000b). Arnold-Schild et al. (1999) have also observed similar differences between gp96 and hsp70. These differences are not inconsistent with our present report pointing to a single receptor for the four HSPs. They simply suggest that the various HSPs interact with a single receptor with widely differing affinities. Castellino et al. (2000) have recently demonstrated re-presentation of hsp70-chaperoned peptides by APCs through receptor-mediated uptake and have

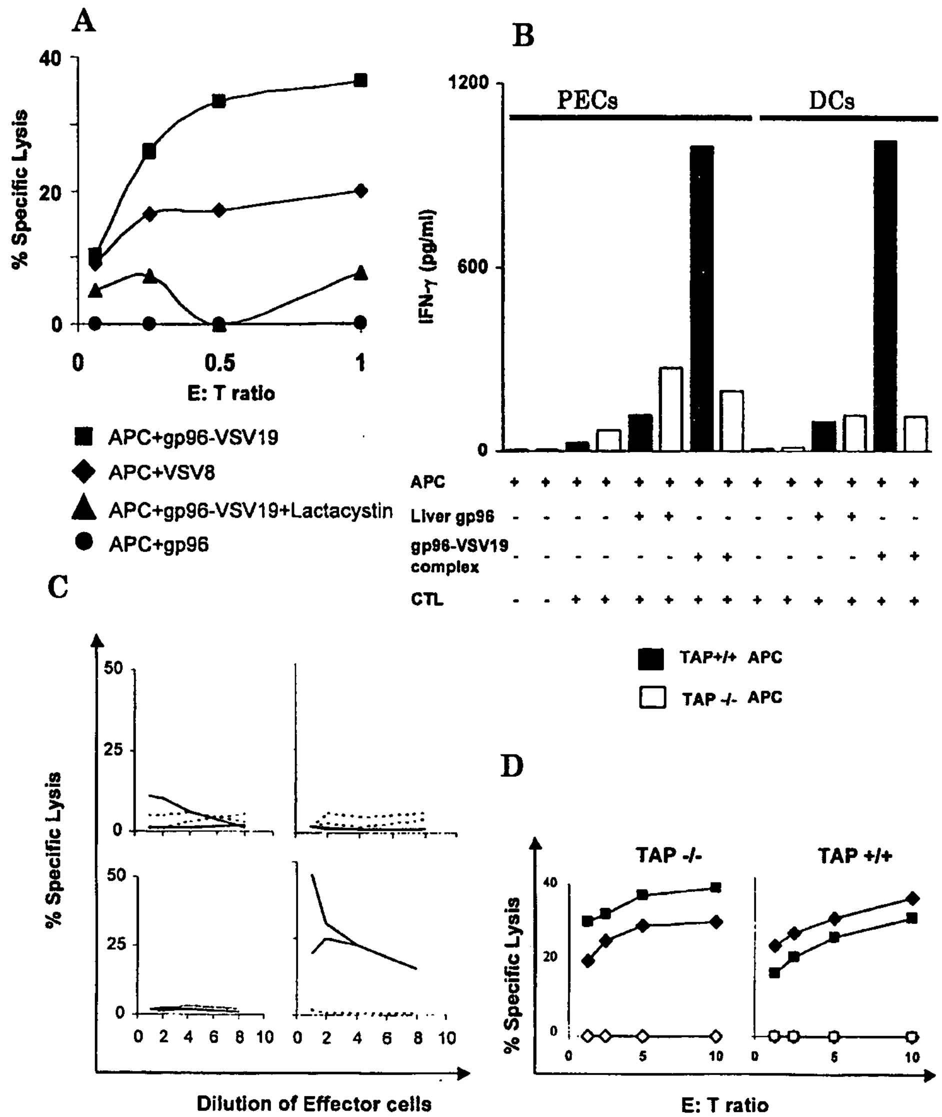

Figure 6. Re-Presentation of gp96-Chaperoned Peptides Follows the Classical Endogenous Antigen Presentation Pathway

(A) Requirement of proteasomes. Peritoneal macrophage (1×10^6) were either treated or untreated with lactacystin (100 μM) for 2 hr at room temperature and then pulsed with gp96-VSV19 complex (40 μg/ml) or gp96 alone for another 2 hr at 37°C. The cells were labeled with chromium and used as targets against VSV8-specific CTLs.

(B) Requirement of TAP as measured in vitro. Peritoneal macrophage or BM-DCs (as indicated) from $TAP^{+/+}$ or $TAP^{-/-}$ mice were cultured with gp96 (40 μg/ml) from liver or gp96-VSV19 complex (40 μg/ml) and VSV8-specific CTL line for 20 hr at 37°C. Culture supernatants were tested for the presence of IFN-γ (pg/ml) as a marker for CTL stimulation.

(C) Requirement of TAP as measured in vivo. Gp96-VSV19 complex (50 μg gp96) was injected intraperitoneally. After 10 days, spleens were removed, and cells were cultured in vitro with VSV8. The lymphocyte cultures were tested for their ability to lyse EL4 cells (dotted line) or EL4 cells pulsed with VSV8 peptide (solid line). Each line represents one mouse.

(D) $TAP^{-/-}$ mice can generate VSV8-specific CTLs. $TAP^{+/+}$ and $TAP^{-/-}$ mice were immunized with VSV8 (100 μg) in incomplete Freund's adjuvant, and splenocytes of immunized mice were tested for their ability to lyse EL4 cells (open symbols) or VSV8-pulsed EL4 cells (closed symbols). Two mice were tested in each group, and data for both mice are shown.

suggested the existence of receptors of different affinity classes for single HSPs. That argument is biologically cogent but is not supported by our present data.

The question of single or multiple receptors cannot be addressed fully without taking into consideration one other consequence of HSP-APC interaction. We have focused our attention thus far on the ability of HSPs to bind the cognate receptors on APCs, for the HSP-peptide complexes to be internalized, and for the peptides chaperoned by them to be re-presented by the MHC class I molecules of the APCs. However, HSP-APC interaction has an additional consequence: engagement of APCs by HSPs activates them to release a number of cytokines and to cause expression of antigen-presenting and costimulatory molecules on them (Basu et al., 2000). In the case of DCs, these phenomena may be grouped into the category of a maturation signal. Are the receptors involved in re-presentation the same as are necessary for the innate aspects of the HSP-APC interaction? Our present studies do not shed light on this question. It is unclear if the CD91 is a signaling receptor (Goretzki and Mueller, 1998) or if HSPs interact with other signaling receptors to mediate the release of cytokines and related phenomena. Preliminary data from our laboratory suggest that the scavenging receptor CD36 is involved in activation of APCs by HSPs to release cytokines and chemokines (Panjwani et al., 2000).

A number of APCs have been analyzed for their ability to re-present HSP-chaperoned peptides. These include PEMs, immature and mature BM-DCs, and RAW264.7 cells. Somewhat surprisingly, we did not observe much heterogeneity among the APCs in their ability to re-present HSP-chaperoned peptides. Within the limits of our ability to quantitate the re-presenting ability, primary macrophages, a macrophage line, and immature and mature DCs were all able to re-present HSP-chaperoned peptides, although the BM-DCs appeared to re-present with a detectably higher efficiency. This observation is partly at variance with the recent report by Singh-Jasuja et al. (2000), who showed a dimunition of gp96 binding in the BM-DCs as a function of their differentiation. The most likely basis for the discrepancy between our results and those of Singh-Jasuja et al. lies in the fact that we have used freshly isolated cultures (6 day) of bone marrow DCs as immature DCs and LPS-stimulated DCs as matured DCs. In contrast, Singh-Jasuja et al. have used long-term cultures of bone marrow DCs without LPS stimulation as matured DCs. Obviously, culture conditions can have profound influence on the status of DCs. It is also conceivable that the difference arises simply from the sensitivity of the specific reagents used (gp96-FITC) by Singh-Jasuja et al. (2000) and in the present study. Our functional studies using the re-presentation assay show that mature DCs are at least as efficient as immature DCs in re-presenting gp96-chaperoned peptides.

Once the HSP-peptide complex binds to the receptor, peptides chaperoned by the HSPs must enter the APC along with the HSP. The studies reported here address the downstream events solely with respect to gp96, in the assumption that, if all HSPs enter through the same portal, the downstream events must be identical or similar for peptides chaperoned by each of them. Our observations show that the peptides go from the endosome to the cytosol to the ER and then to the secretory pathway to be re-presented on the surface. The transit through the cytosol is established through the proteasome requirement as well as through the apparent TAP requirement of re-presentation. There is no known mechanism for transit of molecules from vesicular to soluble compartment, although precedents certainly exist (Chiang et al., 1989). Exploration of this pathway shall without doubt open a new window into our understanding of the intracellular traffic of proteins. Castellino et al. (2000) have reported recently on the events as they occur downstream of receptor-mediated uptake of hsp70-peptide complexes by APCs. Our observations with a different HSP (gp96) are entirely consistent with that version of events and buttress the notion that the same portal of entry is used by all the peptide-chaperoning HSPs for re-presentation.

All the studies on HSP-mediated peptide re-presentation have relied thus far on PEMs and, more recently, BM-DCs as the APCs. The demonstration that a permanent, easily available, and easily cultured cell line of macrophage origin can re-present HSP-chaperoned peptides is an important contribution of this study. The RAW264.7 line is of the d haplotype and, as such, of limited utility; however, its many variants expressing a wide array of restriction elements of mouse and human origin are under construction and should become powerful tools for biochemical, cell biological, and immunological analysis of HSP-mediated re-presentation.

Finally, a comment on the biological functions of the HSP receptor is in order. We have reported recently that HSPs are released from cells as a result of necrotic death but not of apoptotic cell death (Basu et al., 2000). In this context, the identification of CD91 as the gp96 receptor lead us to suggest CD91 to be a sensor of necrotic cell death. Among the four HSPs tested here and, indeed, among all HSPs, hsp90 is the most abundant on a per cell basis. It is also among the most primitive HSPs. In this regard, we have been repeatedly impressed with the observation that hsp90 is the most potent competitive inhibitor for binding of gp96 to APCs (Binder et al., 2000b) and for re-presentation of gp96-chaperoned peptides (this study). Viewing these observations and considerations together, it is our opinion that hsp90, more than other HSPs, is the primary indicator of cell death and that CD91 is perhaps primarily a receptor for hsp90. Gp96, hsp70, and CRT (and presumably hsp110, an hsp70 family member) are able to use CD91 as their receptor, by virtue of their obvious (in the case of gp96) and nonobvious (in the case of others) homologies with hsp90. Clearly, this idea requires further experimental exploration.

Experimental Procedures

Mice, Cells, and Reagents

C57Bl/6, BALB/c, and *TAP*$^{-/-}$ mice were obtained from Jackson Laboratories. Bone marrow-derived DCs were generated from the femurs and tibia of C57BL/6 mice. The bone marrow was flushed out, and the leukocytes were obtained and cultured, as described (Lutz et al., 1999), in complete RPMI1640 with 10% heat-inactivated FCS and 20 ng/ml GMCSF (Endogen, Inc., Woburn, MA) for 6 days. On day 3, fresh media with GMCSF was added to the plates for the

day 6 cultures. Macrophages were obtained from PEMs of pristaned mice by positive selection for CD11b$^+$ cells (Miltenyi Biotech, Inc.). RAW264.7 was a gift of Dr. Christopher Nicchitta. A20.25 was a gift of Dr. Lawrence Kwak. All other cell lines were obtained from ATCC. Proteasome inhibitor Lactacystin was purchased from Kamiya, Inc. (Japan). Anti-CD91 antibody (clone 5A6) was purchased from PROGEN (Heidelberg). Anti-hsp70 (clone N27F3) and anti-PDI (clone 1D3) antibodies were purchased from StressGen (Victoria, Canada).

Purification of HSPs
HSPs were purified as described (Srivastava, 1997; Basu and Srivastava, 1999). All buffers used for purifications were prepared with endotoxin-free water (Nanopure Infinity UV/UF, Barnstead/Thermolyne, Dubuque, IA), and all glasswares used for purification were cleaned with endotoxin-free water and baked in a 400°F oven (Gruenberg, Williamsport, PA). The HSP-containing fractions were identified by immunoblots.

Conjugation of Proteins to FITC and Staining of Cells
Purified proteins were conjugated to FITC, using the FluoroTag FITC conjugation kits (SIGMA) as per the manufacturer's protocol. Conjugation was confirmed by a 2 kDa increase in molecular weight by SDS-PAGE and by immunoblotting with an anti-FITC monoclonal antibody. Incubations of indicated amounts of FITC-tagged proteins and cells were done in the presence of 1% nonfat dry milk (Carnation) in PBS for 20 min at 4°C. After repeated washing, cells were analyzed by flow cytometry (Becton Dickenson, La Jolla, CA). Cells were also labeled with propidium iodide just before FACScan analysis. Cells staining positive for propidium iodide were gated out of the events. No differences were observed in the binding of HSPs to Mac-1$^+$ cells from pristaned or nonpristaned mice.

Complexing In Vitro of Peptide to HSPs
This was carried out as previously described (Blachere et al, 1997). HSPs were mixed with VSV19 or AH1/19 in a 50:1 peptide to protein molar ratio in 0.7 M NaCl in Na-phosphate buffer and heated at 50°C for 10 min then incubated at room temperature for 30 min. Excess free peptide was removed with PBS, using centricon 10 (Amicon, Inc., Beverly MA).

Purification of CD11b$^+$ Cells
CD11b$^+$ cells were selected using the MACS columns and protocols supplied by Miltenyi Biotec, Inc. (Auburn, CA). CD11b antibody, supplied as CD11b MicroBeads, was purchased from Miltenyi Biotec, Inc., and has been demonstrated not to activate CD11b$^+$ cells with regard to the markers tested in this experiment.

In Vitro Re-Presentation Assay
CD11b$^+$ peritoneal exudate cells (1×10^6) were pulsed with HSPs purified from liver or HSP-peptide complex generated in vitro, and relevant CD8$^+$ T cells (VSV8-specific CTL line or AH1-specific CTL clones, as indicated) were added to the cultures. The assay was carried out in 250 μl volume in 96-well plates with RPMI medium containing 5% FCS at 37°C for 20 hr. Culture supernatants were harvested and tested for the presence of IFN-γ release by ELISA (Endogen, Inc., Woburn, MA).

Induction of Cytotoxic T Cells
C57BL/6 mice were immunized intraperitoneally with 50 μg of gp96 complexed with VSV19 peptide. At 10 days later, recipient spleens were removed, and splenocytes were stimulated with VSV8 synthetic peptide at 1 μM concentration. After 5 days, cultures were tested for cytotoxicity in a chromium release assay using EL4 cells alone and EL4 cells pulsed with VSV8 peptide as targets.

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References

Arnold-Schild, D., Hanau, D., Spehner, D., Schmid, C., Rammensee, H.G., de la Salle, H., and Schild, H. (1999). Receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. J. Immunol. *162*, 3757–3760.

Basu, S., and Srivastava, P.K. (1999). Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. J. Exp. Med. *189*, 797–802.

Basu, S., Binder, R.J., Suto, R., Anderson, K.M., and Srivastava, P.K. (2000). Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a maturation signal to dendritic cells and activate the NFκB pathway. Int. Immunol. *12*, 1539–1546.

Bevan, M.J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J. Exp. Med. *143*, 1283–1288.

Binder, R., Han, D.K., and Srivastava, P.K. (2000a). CD91: a receptor for heat shock protein gp96. Nature Immunol. *2*, 151–155.

Binder, R.J., Harris, M., Menoret, A., and Srivastava, P.K. (2000b). Saturation, competition and specificity in interaction of heat shock proteins (hsp) gp96, hsp90, and hsp70 with CD11b+ cells. J. Immunol. *165*, 2582–2587.

Blachere, N.E., Li, Z., Chandawarkar, R.Y., Suto, R., Jaikaria, N.S., Basu, S., Udono, H., and Srivastava, P.K. (1997). Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. J. Exp. Med. *186*, 1315–1322.

Breloer, M., Marti, T., Fleischer, B., and von Bonin, A. (1998). Isolation of processed, H-2Kb-binding ovalbumin-derived peptides associated with the stress proteins HSP70 and gp96. Eur. J. Immunol. *28*, 1016–1021.

Castellino, F., Boucher, P.E., Eichelberg, K., Mayhew, M., Rothman, J.E., Houghton, A.N., and Germain, R.N. (2000). Receptor-mediated uptake of antigen/heat shock protein complex results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J. Exp. Med. *191*, 1957–1964.

Chiang, H.L., Terlecky, S.R., Plant, C.P., and Dice, J.F. (1989). A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science. *246*, 382–385.

Goretzki, L., and Mueller, B.M. (1998). Low-density-lipoprotein-receptor-related protein (LRP) interacts with a GTP-binding protein. Biochem. J. *336*, 381–386.

Huang, A.Y., Gulden, P.H., Woods, A.S., Thomas, M.C., Tong, C.D., Wang, W., Engelhard, V.H., Pasternack, G., Cotter, R., Hunt, D., et al. (1996). The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. Proc. Natl. Acad. Sci. USA *93*, 9730–9735.

Ishii, T., Udono, H., Yamano, T., Ohta, H., Uenaka, A., Ono, T., Hizuta, A., Tanaka, N., Srivastava, P.K., and Nakayama, E. (1999). Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. J. Immunol. *162*, 1303–1309.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rossner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods *223*, 77–92.

Ohashi, K., Burkart, V., Flohe, S., and Kolb, H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J. Immunol. *164*, 558–561.

Panjwani, N.N., Popova, L., Febbraio, F., and Srivastava, P.K. (2000). The CD36 scavenger receptor as a receptor for gp96. Cell Stress and Chaperones *5*, 391.

Prodromou, C., Roe, S.M., O'brien, R., Ladbury, J.E., Piper, P.W., and Pearl, L.H. (1997). Identification and structural characterization

of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell *90*, 65–75.

Sandberg, J.K., Chambers, B.J., Van Kaer, L., Karre, K., and Ljunggren, H.G. (1996). TAP1-deficient mice select a CD8+ T cell repertoire that displays both diversity and peptide specificity. Eur. J. Immunol. *26*, 288–293.

Singh-Jasuja, H., Scherer, H.U., Hilf, N., Arnold-Schild, D., Rammensee, H.G., Toes, R.E., and Schild, H. (2000). The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. Eur. J. Immunol. *30*, 2211–2215.

Srivastava, P.K. (1982). Studies on cell surfaces during normal and abnormal growth: purification of a tumor-associated antigen and a tumor rejection antigen from a rat hepatoma. PhD thesis, Osmania University, Hyderabad, India.

Srivastava, P.K. (1994). Heat shock proteins in immune response to cancer: the fourth paradigm. Experientia *50*, 1054–1060.

Srivastava, P.K. (1997). Purification of heat shock protein-peptide complexes for use in vaccination against cancers and intracellular pathogens. Methods *12*, 165–171.

Srivastava, P.K., and Das, M.R. (1984). The serologically unique cell surface antigen of Zajdela ascitic hepatoma is also its tumor-associated transplantation antigen. Int. J. Cancer. *33*, 417–419.

Srivastava, P.K., DeLeo, A.B., and Old, L.J. (1986). Tumor rejection antigens of chemically induced sarcomas of inbred mice. Proc. Natl. Acad. Sci. USA *83*, 3407–3411.

Srivastava, P.K., Udono, H., Blachere, N.E., and Li, Z. (1994). Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics *39*, 93–98.

Srivastava, P.K., Menoret, A., Basu, S., Binder, R.J., and McQuade, K.L. (1998). Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity *8*, 657–665.

Stebbins, C.E., Russo, A.A., Schneider, C., Rosen, N., Hartl, F.U., and Pavletich,N.P. (1997). Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell *89*, 239–250.

Suto, R., and Srivastava, P.K. (1995). A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. Science *269*, 1585–1588.

Udono, H., and Srivastava, P.K. (1993). Heat shock protein 70-associated peptides elicit specific cancer immunity. J. Exp. Med. *178*, 1391–1396.

Udono, H., and Srivastava, P.K. (1994). Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90 and hsp70. J. Immunol. *152*, 5398–5403.

Udono, H., Levey, D.L., and Srivastava, P.K. (1994). Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells in vivo. Proc. Natl. Acad. Sci. USA *91*, 3077–3081.

Ullrich, S.J., Robinson, E.A., Law, L.W., Willingham, M., and Appella, E. (1986). A mouse tumor-specific transplantation antigen is a heat shock-related protein. Proc. Natl. Acad. Sci. USA *83*, 3118–3125.

Wang, X.Y., Chen, X., Oh, H.J., Repasky, E., Kazim, L., and Subjeck, J. (2000). Characterization of native interaction of hsp110 with hsp25 and hsc70. FEBS Lett. *465*, 98–102.

Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E., and Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. Science. *272*, 1606–1614.